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UREA PARTHENOGENETICALLY ACTIVATES THE CORTICAL REACTION AND ELONGATION OF MICROVILLI IN EGGS OF THE SEA URCHIN, *STRONGYLOCENTROTUS PURPURATUS*

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Abstract

Isotonic urea is believed to activate sea urchin eggs by triggering event(s) that normally follow cortical granule secretion at fertilization, particularly surface perturbations that result in elongation of microvilli (Mazia *et al.*, 1975). However, Moser (1940) reported that urea triggered the cortical reaction. Transmission electron microscopy showed that unfertilized *Strongylocentrotus purpuratus* eggs discharge their cortical granules in isotonic urea (containing 1.0 to 0.1 m*M* $CaCl_2$ or 25 m*M* EGTA) to form incipient fertilization envelopes and hyaline layers. These investments quickly disperse in urea. Elongation of microvilli follows cortical granule discharge. Urea-activated eggs can be fertilized after return to sea water and fail to elevate fertilization envelopes but do form hyaline layers. Hyalin must be secreted from a secondary reservoir in these eggs, since the cortical granule store is discharged during the prior urea activation. Cortical granule secretion and elongation of microvilli do not occur in urea plus 10 m*M* $CaCl_2$. These eggs form normal fertilization envelopes and hyaline layers when fertilized after return to sea water. Our results show that: (1) urea triggers an early event in sea urchin egg activation that stimulates cortical granule secretion; (2) cortical granule discharge precedes elongation of microvilli in urea-activated eggs as it does during normal fertilization; and (3) reduction or removal of external calcium is required for activation by urea.

Introduction

Parthenogenetic agents, including isotonic non-electrolytes such as urea, have been used to study the sequence of events and causal relationships responsible for the activation of sea urchin eggs during fertilization (reviewed by: Loeb, 1913; Lillie, 1919; Allen, 1958; Epel, 1978; Schuel, 1978; Jaffe, 1980). On the basis of a scanning electron microscopic study performed on *Strongylocentrotus purpuratus* and *Lytechinus pictus*, it was suggested that urea bypassed the cortical reaction and activated the sea urchin egg by releasing a repressor component from its surface (plasma membrane and/or vitelline layer) and by inducing elongation of microvilli (Mazia *et al.*, 1975). Ammonia activation, which does not induce the cortical reaction (Loeb, 1913; Steinhardt and Mazia, 1973; Epel *et al.*, 1974), also was reported to promote elongation of microvilli (Mazia *et al.*, 1975). The concept advanced by Mazia's group appeared to fit with observations that: (1) elongation of microvilli normally occurs subsequent to secretion of the cortical granules during fertilization (Schroeder, 1979); (2) detachment of the vitelline layer from the egg's plasma membrane, a step

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in the assembly of the fertilization envelope (Schuel, 1978), is promoted by a protease secreted by the cortical granules (Longo and Schuel, 1973; Schuel *et al.*, 1973; Vacquier *et al.*, 1973); (3) urea removes the vitelline layer from unfertilized eggs (Moore, 1930); and (4) ammonia releases a surface glycoprotein that results in derepression of the egg's metabolism as normally takes place during fertilization (Johnson and Epel, 1975). The putative role of the release of repressor protein from the surface of ammonia-activated egg has been refuted (Carroll and Epel, 1981). Moreover, Mazia's group did not consider the possibility that urea can induce the cortical reaction, and that elongation of microvilli might be related to cortical granule exocytosis rather than to surface modifications. Urea had previously been shown to induce secretion of the cortical granules during parthenogenetic activation of *Arbacia* eggs (Moser, 1940).

The present study was undertaken to re-examine the effects of isotonic urea on the surface morphology of unfertilized *Strongylocentrotus purpuratus* eggs by means of transmission light and electron microscopy. A preliminary account has been presented previously (Schuel and Dandekar, 1981).

Materials and Methods

Specimens of the sea urchin *Strongylocentrotus purpuratus* were obtained from Pacific Bio-Marine Laboratories (Venice, CA) and maintained at 12–15°C in a marine aquarium (Aquarium Systems, Inc., Wickliffe, OH). Gametes were collected and stored as described previously (Schuel and Schuel, 1981). Only batches of eggs that yielded 95–100% fertilization in test insemination (0.1 ml eggs per 5 ml sea water plus 0.1 ml of 1% sperm) were used in this study. Experimental cultures were incubated at 15°C.

Artificial sea water was prepared from Instant Ocean salt mixture (Aquarium Systems, Inc.) and filtered through a 0.45μ Millipore filter. Calcium-free sea water containing 25 m*M* EGTA (ethyleneglycol-bis(β-amino ethyl ether)N,N′-tetra acetic acid) was prepared according to Detering *et al.* (1977). Isotonic urea (1.0 *M*) was prepared in deionized water, 10 m*M* $CaCl_2$, or 25 m*M* EGTA adjusted to pH 8.0 with NaOH. EGTA was obtained from Sigma Chemical Co., St. Louis, MO.

Unfertilized eggs were activated parthenogenetically by brief exposure to isotonic urea (Moser, 1940; Mazia *et al.*, 1975). Egg suspensions (1.0 ml) were added to 9.0 ml of urea and incubated for 60 sec. The eggs were then sedimented by gentle centrifugation (IEC Clinical Centrifuge) and the supernatant discarded. The eggs were resuspended in urea (to 10 ml), incubated for another 60 sec, sedimented again by centrifugation, and finally resuspended in sea water. This treatment took about 3 min. After exposure to the urea solutions, the eggs were inseminated and cultured in sea water. In some experiments eggs were observed for up to 5 minutes during a single continuous treatment with 9 parts isotonic urea plus 1 part sea water.

For morphological analysis, eggs were fixed with 3% glutaraldehyde in sea water. They were then processed for examination by transmission light and electron microscopy using previously described procedures (Longo and Anderson, 1972). Thin sections stained with uranyl acetate and lead citrate were examined with a JEOL-100B electron microscope. Thick sections stained with toluidine blue were examined by light microscopy.

Results

Live *Strongylocentrotus* eggs were observed by light microscopy during and following treatment with isotonic urea. Thin fertilization envelopes elevate from the

surface of unfertilized eggs in the urea solution. Upon continued exposure to urea the fertilization envelopes recede toward the egg surface and become thinner, until in most cases no vestige of the fertilization envelope can be seen. These results are consistent with previous observations that urea parthenogenetically activates the cortical reaction in *Arbacia* eggs (Moser, 1940). When urea-activated eggs (two 60-sec washes) are returned to sea water they are indistinguishable from control eggs incubated in sea water. Urea-activated eggs can be fertilized. However, following insemination none of these eggs lift fertilization envelopes, but most form hyaline layers. Control eggs form normal fertilization envelopes and hyaline layers upon fertilization. Eggs fertilized following urea activation divide and develop at the same time as controls. About 10% of the urea-treated eggs fail to form hyaline layers after subsequent fertilization. These zygotes divide to form unorganized grape-like clusters of blastomeres during cleavage. These findings confirm previous observations by Moore (1930).

The effects of urea treatment on the surface morphology of *Strongylocentrotus* eggs was determined by light (data not shown) and electron microscopic (Figs. 1 and 2) analysis of fixed and sectioned specimens. Cortical granules are located subjacent to the plasma membrane in unfertilized (control) eggs (Fig. 1A). The vitelline layer is attached to the outer surface of the egg's plasma membrane, and short microvilli are present at the egg surface. Upon exposure to isotonic urea (9 parts plus 1 part sea water) the cortical reaction is triggered and results in lifting of the fertilization envelope (Fig. 1B). Patches of "hyalin-like" material are seen in the perivitelline space, but an organized hyaline layer does not form. The fertilization envelopes begin to fragment and disperse upon continued exposure to urea (data not shown). Examination of eggs returned to sea water after two 60-sec washes in isotonic urea reveals that the treatment completely removes the fertilization envelopes (Fig. 2A). Elongate microvilli are prominent features at the surface of these eggs. External investments (vitelline layer/fertilization envelope and hyaline layer) can not be detected outside of the eggs. After these eggs are fertilized, they form normal hyaline layers but do not form fertilization envelopes (Fig. 2B).

Several other aspects of the responses of urea-activated eggs were observed. When eggs are suspended in urea the fertilization envelope appears to lift simultaneously from the entire circumference of the activated eggs. Examination of fixed and sectioned specimens indicates that in each individual urea-activated egg the cortical reaction is at the same stage around the entire circumference (data not shown). By contrast, during normal fertilization the cortical reaction and the elevation of the fertilization envelope start at the site of attachment of the fertilizing sperm and spread around the surface of the egg (Moser, 1939a; Anderson, 1968). The incidence of eggs in the population that show a cortical reaction increases with exposure time to urea (Fig. 3). The data are presented in the form of a first order decay plot of unreacted eggs *vs* exposure time, from which the half time for urea activation can be estimated to be about 90 sec.

The release of calcium from internal stores is believed to play a critical role in the initiation of cortical granule exocytosis in sea urchin eggs during fertilization and upon parthenogenetic activation (reviewed by: Epel, 1978; Schuel, 1978; Jaffe, 1980). Accordingly we studied the effects of calcium on the parthenogenetic induction of the cortical reaction by urea. The normal calcium concentration of sea water is 10 m*M* (Cavanaugh, 1964). In the urea-activation experiments described above (Fig. 2), calcium is reduced to 1.0 m*M* in the first wash and 0.1 m*M* in the second wash. The urea solutions used by Mazia's group (1975) to induce elongation of microvilli contained 0.1 m*M* calcium. We found that when 25 m*M* EGTA is added

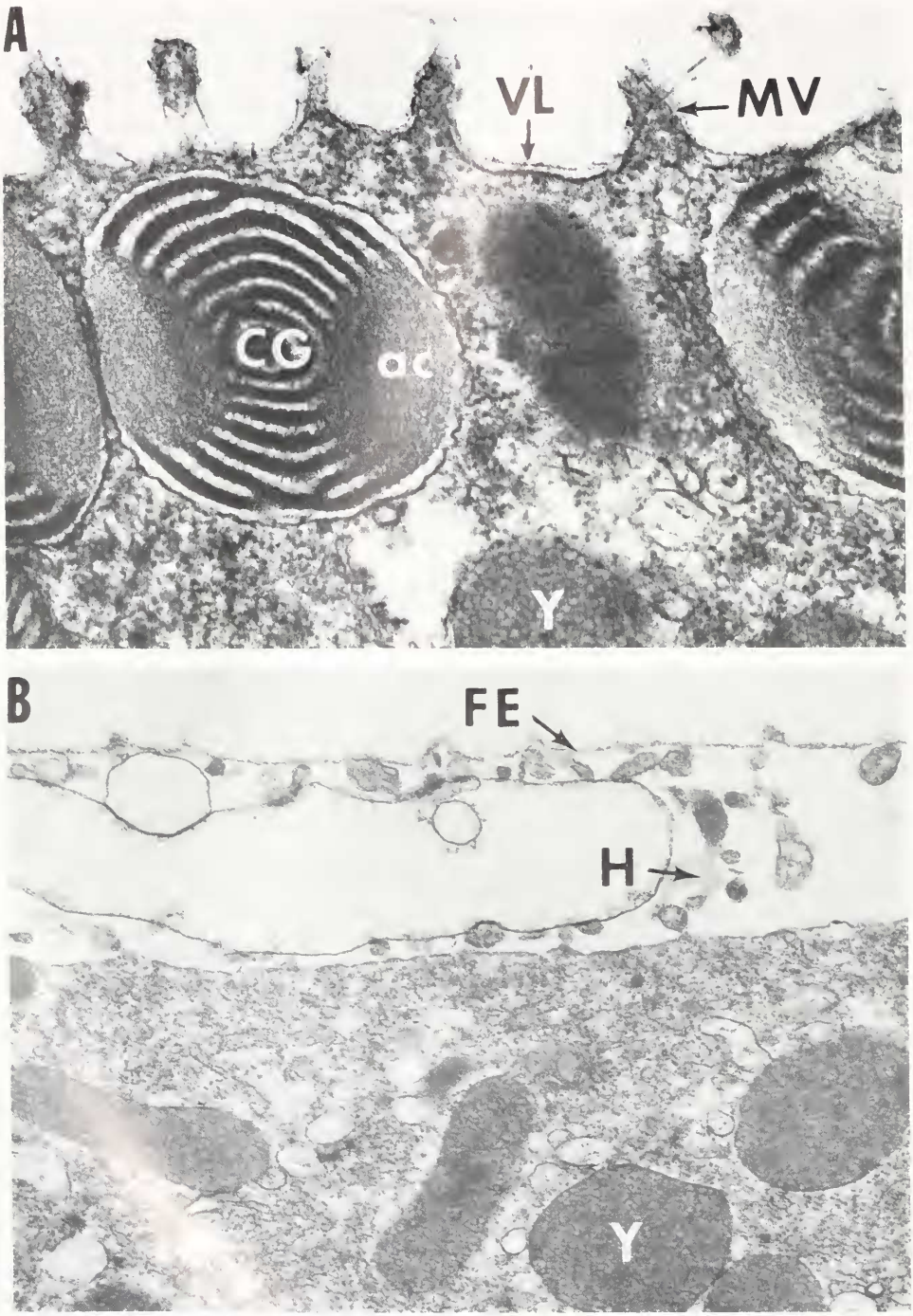

FIGURE 1. Electron micrographs showing parthenogenetic induction of the cortical reaction in unfertilized *Strongylocentrotus* eggs by isotonic urea.

A: Control egg in sea water. Cortical granules (CG) located just below the egg's plasma membrane show the amorphous (ac) and electron-dense spiral lamellae components characteristic of this species.

to the isotonic urea, cortical granule discharge occurs followed by elongation of microvilli as described above. Control eggs incubated under similar conditions in calcium-free sea water containing 25 m*M* EGTA do not show a cortical reaction (data not shown). Under these conditions EGTA reduces the free calcium in the culture solutions to below 10^{-7} *M* (Portzehl *et al.*, 1964). Conversely, cortical granule secretion and elongation of microvilli do *not* take place when unfertilized eggs are exposed to isotonic urea containing 10 m*M* calcium (Fig. 4A). When these eggs are fertilized after return to sea water, they undergo a normal cortical reaction to produce fertilization envelopes and hyaline layers (Fig. 4B). The thickened tri-laminar fertilization envelope shows sharp "tent-like" projections indicative of structuralization by secreted cortical granule contents in *Strongylocentrotus* (Veron *et al.*, 1977; Schuel *et al.*, 1982). These observations confirm previous findings by Moore (1930) that inclusion of calcium in the urea solutions protects the egg's capacity to form a fertilization envelope upon insemination after return to sea water.

Discussion

The results of the present study show that, contrary to previous suggestions (Mazia *et al.*, 1975), urea does not mimic the presumed effects of ammonia in activating sea urchin eggs by triggering events that normally occur subsequent to the cortical reaction. Instead urea triggers discharge of the cortical granules. Furthermore, exocytosis of the cortical granules precedes elongation of microvilli in urea-activated eggs just as it does during normal fertilization. These findings confirm and extend earlier observations by Moser (1940). Although we did not examine their effects, Moser also noted that other non-electrolytes (glycerol, thiourea, and sucrose) elicited the same kind of visible cortical response as urea.

Elongation of microvilli during fertilization or upon parthenogenetic activation is a complex process that depends in part upon the insertion of the limiting membrane of the discharged cortical granules into the egg's original plasma membrane as a result of exocytosis (Schroeder, 1979) as well as the polymerization of actin in the cortex to form bundles of microfilaments (Burgess and Schroeder, 1977; Carron and Longo, 1982). Urea appears to mimic other parthenogenetic treatments such as hypertonic sea water (Sachs and Anderson, 1970) and calcium ionophore A23187 (Chambers and Hinkley, 1979; Carron and Longo, 1982) which induce elongation of microvilli as sequalae to cortical granule exocytosis. Elongation of microvilli in the absence of cortical granule exocytosis can be induced by application of hydrostatic pressure immediately after insemination (Chase, 1967; Hylander and Summers, 1982) and by treating unfertilized eggs with papain (Spiegel and Spiegel, 1977). The belief that ammonia and urea induce microvillar elongation in the absence of prior cortical granule exocytosis (Mazia *et al.*, 1975) appears to be erroneous. Other workers who examined ammonia-activated eggs by transmission electron microscopy found that the microvilli do not elongate and the cortical granules do not secrete (Nicotra and Arizzi, 1979; Hylander and Summers, 1981; Carron and Longo, 1982; Schuel and Dandekar, unpublished data). Cortical granule exocytosis is sometimes seen during ammonia activation (Carroll and Epel, 1981). However, Mazia's group (1975) did not determine whether cortical granule exocytosis had occurred in urea or ammonia activated eggs.

The vitelline layer (VL) is closely applied to the outer surface of the plasma membrane. Note the short microvilli (MV). Yolk platelet (Y). 50,000×.

B: Activated egg fixed during exposure to 9 parts isotonic urea and 1 part sea water. The cortical granules have discharged and a thin fertilization envelope (FE) has elevated over the egg surface. A patch of "hyalin-like" material (H) is present in the perivitelline space. Yolk platelet (Y). 33,300×.

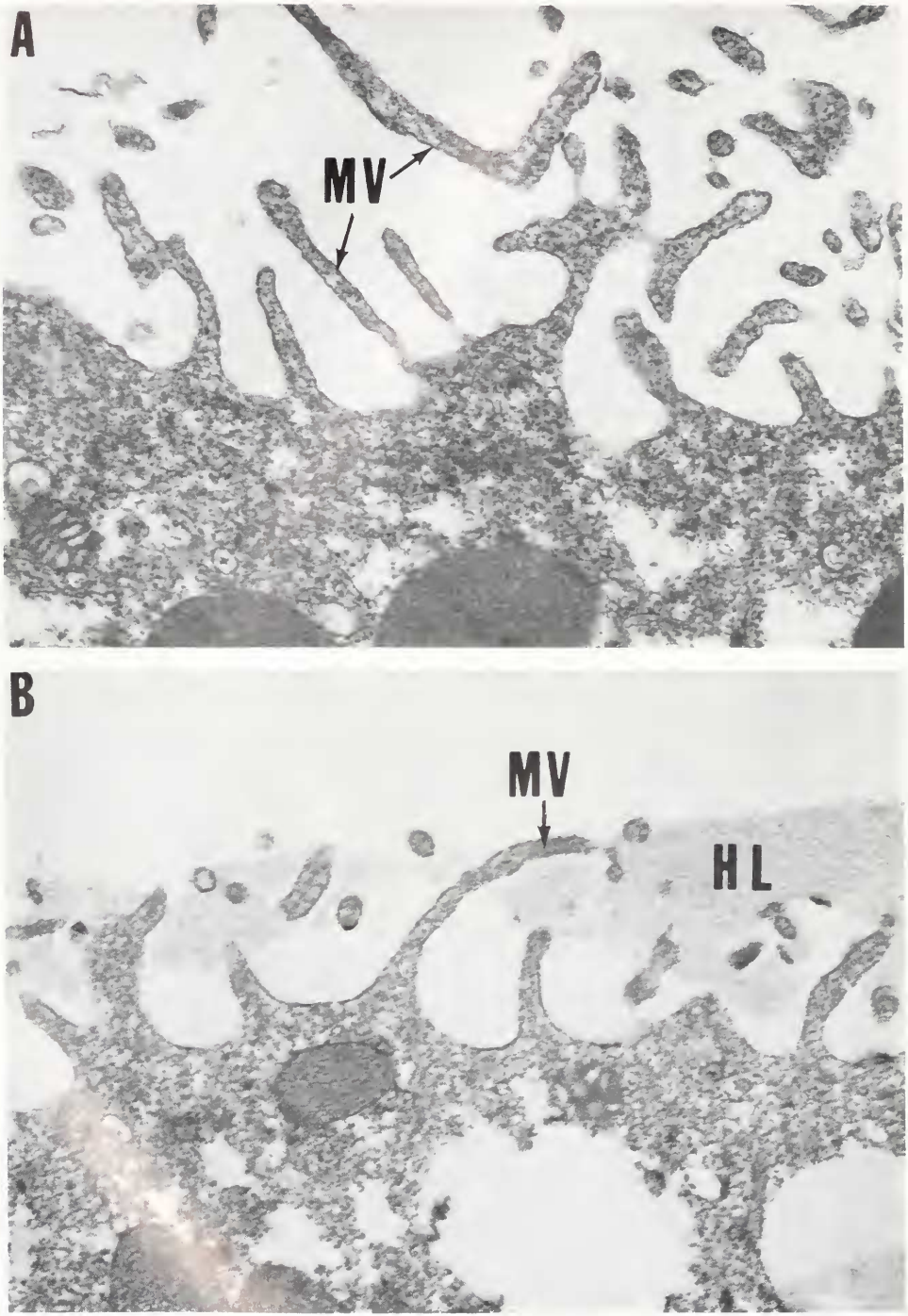

FIGURE 2. Electron micrographs showing the formation of the hyaline layer in urea-activated eggs that are fertilized after return to sea water. 33,300×.

A: Egg washed twice with isotonic urea and fixed immediately after return to sea water. Note the numerous elongate microvilli (MV) and the absence of cortical granules.

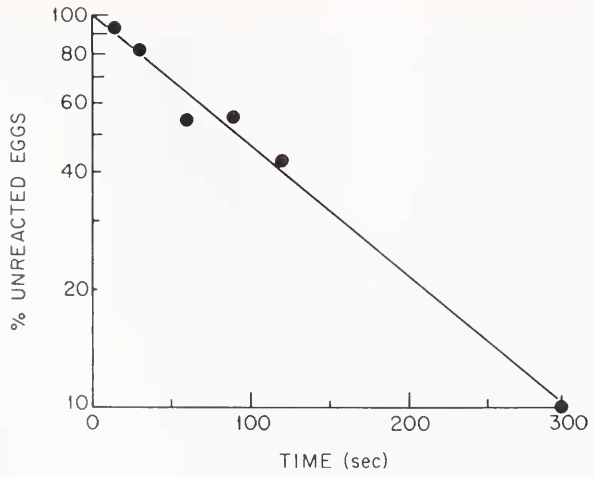

FIGURE 3. Effect of exposure time on incidence of cortical reaction in eggs activated by urea. The eggs (1.0 ml) were exposed to 9.0 ml of isotonic urea and fixed at indicated times. The incidence of reacted and unreacted eggs was scored from thick sections observed by light microscopy.

Morphological (Endo, 1961; Anderson, 1968) and biochemical (Kane, 1970) observations suggest that hyalin, the major structural protein of the hyaline layer (Stephens and Kane, 1970; Citkowitz, 1971), is secreted by the cortical granules during fertilization (reviewed by Schuel, 1978). In addition a secondary cytoplasmic reservoir that normally is slowly released during embryogenesis also is present in unfertilized eggs (Kane, 1973). These concepts have become controversial because McBlaine and Carroll (1980) claimed to show that hyalin is a cryptic protein on the surface of unfertilized eggs. The issue has been resolved by recent immunocytochemical studies using monospecific antibodies against pure hyalin (Hylander, 1981; Hylander and Summers, 1982; McClay and Fink, 1982). They found that hyalin is not detectable on the surface of eggs prior to secretion of the cortical granules, and is sequestered within cortical granules of unfertilized eggs. At the ultrastructural level hyalin is localized to the amorphous component of *Strongylocentrotus* cortical granules (Hylander, 1981; Hylander and Summers, 1982). The secondary hyalin reservoir is stored in small cytoplasmic vesicles (Hylander, 1981; Hylander and Summers, 1982). In the present study the hyaline layer formed by eggs that are fertilized subsequent to urea activation must have been secreted by the secondary reservoir, since the cortical granule store was discharged and dispersed while the eggs were being pretreated with urea. Hence this treatment could be used to collect hyalin from its two cytoplasmic reservoirs for further study.

Isotonic urea has been used to remove the vitelline layer from unfertilized sea urchin eggs (Moore, 1930) and the soft (non-cross-linked) fertilization envelope from fertilized eggs prior to the completion of hardening (Schuel *et al.*, 1982). When urea is applied to unfertilized eggs, it induces both the cortical reaction as well as the dispersal of the elevated fertilization envelope, and does not simply remove the vitelline layer as previously believed (Mazia *et al.*, 1975). Although the urea-activated egg remains receptive to sperm, its plasma membrane has been altered by cortical

B: Urea-activated egg that was fertilized immediately after return to sea water. Fixed 10 min after insemination. Note the elongate microvilli (MV) embedded in the hyaline layer (HL) that invests the egg surface and the absence of the fertilization envelope.

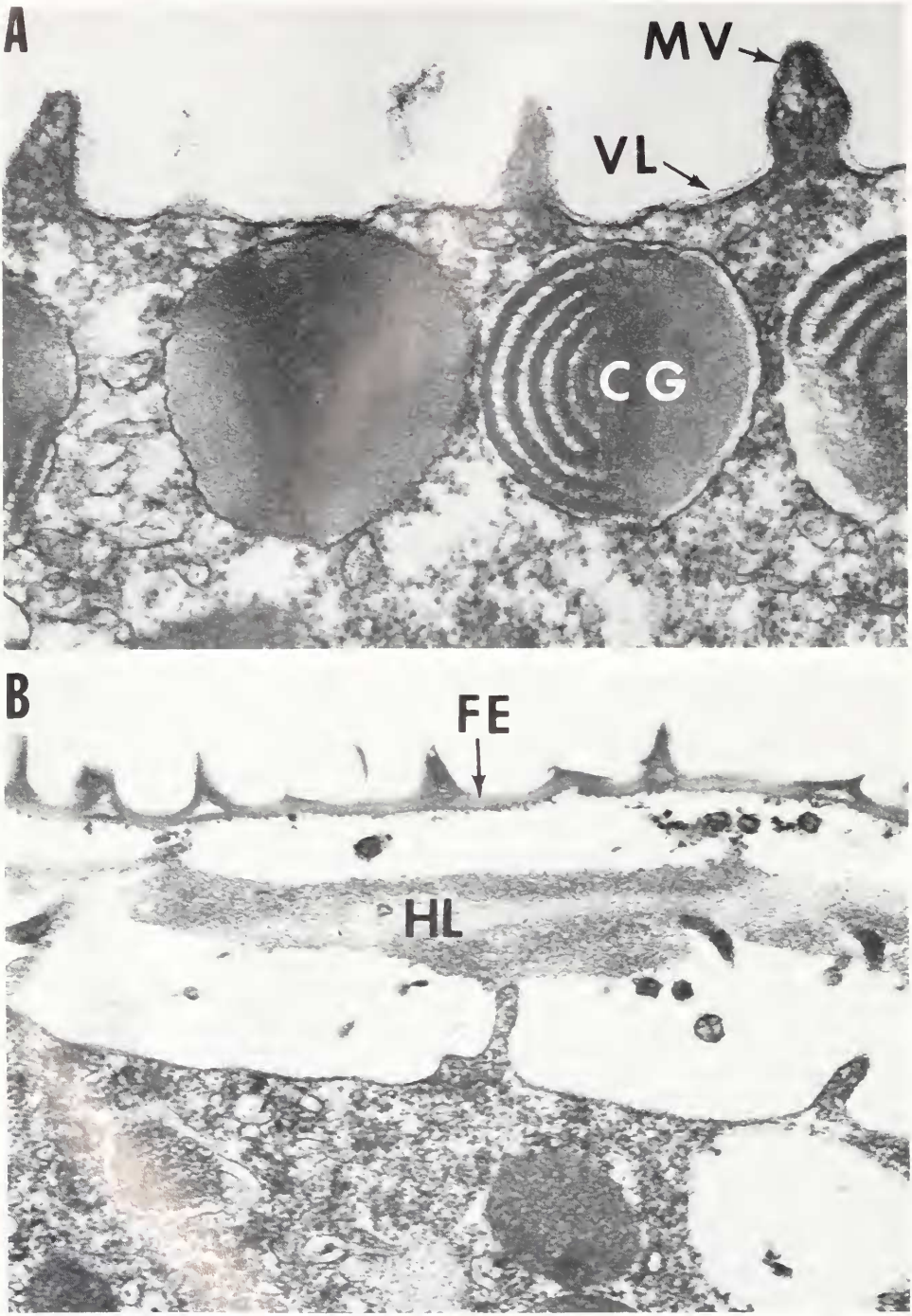

FIGURE 4. Absence of cortical reaction in eggs treated with isotonic urea containing 10 m*M* $CaCl_2$. *A*: Egg washed twice (60 sec each) with isotonic urea containing 10 m*M* $CaCl_2$, and fixed immediately after return to sea water. The cortex of this egg is identical to that of control eggs kept in sea water

granule exocytosis and elongation of microvilli to resemble that of a naked fertilized egg.

The effects of calcium on initiation of cortical granule secretion by urea are paradoxical. Certain other chemical and physical treatments that parthenogenetically trigger the cortical reaction in sea urchins require external calcium (Moser, 1939b). Also, the release of calcium from an internal store is thought to be part of the trigger mechanism for cortical granule exocytosis at fertilization or parthenogenetic activation (Steinhardt *et al.*, 1977; Zucker *et al.*, 1978). Calcium is stored at several sites (vitelline layer, plasma membrane, limiting membranes of cortical granules and other cytoplasmic organelles) in unfertilized eggs (Cardasis *et al.*, 1978), although the identity of the store that is released at fertilization is unknown. Urea appears to trigger the release of calcium from the same store that normally is released at fertilization (Zucker *et al.*, 1978). Yet the results of the present study show that urea elicits cortical granule secretion only when the external calcium is reduced. Taken together these findings possibly suggest that the removal of calcium from binding sites at the egg surface, perhaps the vitelline layer or plasma membrane, may be a prerequisite for the release of an internal store to trigger exocytosis. This feature of the response of sea urchin eggs to urea activation may provide a unique opportunity to study the initial actions of calcium in stimulus-secretion coupling and activation of development. Alternatively it is possible that the calcium level in normal sea water renders the unfertilized egg impermeable to urea and thereby inhibits parthenogenetic activation by the non-electrolyte. Additional work is required to answer these questions.

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(compare with Fig. 1A, above). Note the intact cortical granules (CG), vitelline layer (VL) applied to the egg's plasma membrane and the short microvilli (MV). 50,000×.

B: Isotonic urea-10 m*M* $CaCl_2$ treated egg that was fertilized immediately after return to sea water. Fixed 10 min after insemination. Normal fertilization envelope (FE) and hyaline layer (HL) have been formed. 33,300×.

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